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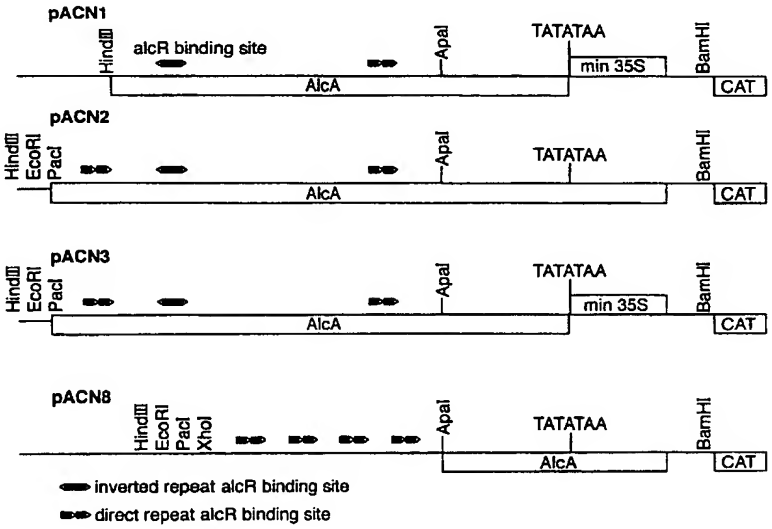
(54) Title: EXPRESSION SYSTEM



(57) Abstract: A chemically-inducible plant gene expression cassette is described. The expression cassette comprises a first promoter operatively linked to the *alcR* regulator protein sequence obtainable from *Aspergillus nidulans* which encodes the AlcR regulator protein, and an inducible promoter obtainable from the *alcA* gene promoter of *Aspergillus nidulans* operatively linked to a target gene, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene and wherein the *alcA* promoter has three or more binding sites for the AlcR protein. According to a preferred embodiment of the invention, the promoter comprises 4 direct repeat sequences for the AlcR protein.

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EXPRESSION SYSTEM

The present invention relates to an expression system for use in plants and in particular to an expression system which utilises an exogenous chemical agent as a control mechanism.

The present invention also relates to plants comprising such an expression system.

The present invention further relates to a chemically-inducible promoter.

Gene expression is controlled by regions upstream (5') of the protein encoding region, commonly referred to as the "promoter". A promoter may be constitutive, tissue-specific, developmentally programmed or inducible.

Manipulation of crop plants to improve characteristics (such as productivity or quality) requires the expression of foreign or endogenous genes in plant tissues. Such genetic manipulation therefore relies on the availability of means to control gene expression as required; for example, on the availability and use of suitable promoters which are effective in plants. It is advantageous to have a choice of a variety of different promoters so that the most suitable promoter may be selected for a particular gene, construct, cell, tissue, plant or environment. A range of promoters are known to be operative in plants.

The term "inducible promoter" includes promoters which may be induced chemically. Particularly useful promoters are promoter sequences which are controlled by the application of an external chemical stimulus. The external chemical stimulus may be an agriculturally acceptable chemical, the use of which is compatible with agricultural practice and is not detrimental to plants or mammals. This allows particular gene expression to be controlled at particular stages of plant growth or development, by the presence or absence of a chemical which can be applied to the plants or seeds, for example by spraying or using known seed coating techniques. These are also known as "gene switch" promoters. Examples of inducible switch systems include the ecdysone switch system as described in our International Publication No. WO 96/37609 or the GST promoter as described in our International Publication Nos WO 90/08826 and WO 93/031294.

Gene switches of this type are known in a wide variety of applications. These include the production of reversible male sterility, a feature which is highly desirable in hybrid plant production as described for instance in WO 90/08830. Other applications of such promoters include their use in the prevention of pre-harvesting sprouting as described in WO 94/03619.

One such example can be found in the fungal organism *Aspergillus nidulans* which expresses the enzyme alcohol dehydrogenase I (ADH1) encoded by the gene *alcA* only when it is grown in the presence of various alcohols and ketones. The induction is relayed through a regulator protein encoded by the *alcR* gene which is constitutively expressed. In the presence of inducer (alcohol or ketone), the regulator protein activates the expression of the inducer. This means that high levels of the ADH 1 enzyme are produced under inducing conditions (i.e. when alcohol or ketone are present). Conversely, the *alcA* gene and its product, ADH 1, are not expressed in the absence of inducer. Expression of *alcA* and production of the enzyme is also repressed in the presence of glucose.

Thus, the *alcA* gene promoter is an inducible promoter, activated by the AlcR regulator protein in the presence of inducer (i.e. by the protein/ alcohol or protein/ ketone combination). The *alcR* and *alcA* genes (including the respective promoters) have been cloned and sequenced (Lockington RA *et al.*, 1985, Gene, 33: 137-149; Felenbok B *et al.*, 1988, Gene, 73: 385-396; Gwynne *et al.*, 1987, Gene, 51: 205-216).

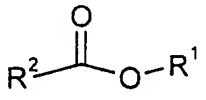
Alcohol dehydrogenase (*adh*) genes have been investigated in certain plant species. In maize and other cereals they are switched on by anaerobic conditions. The promoter region of *adh* genes from maize contains a 300 base pair regulatory element necessary for expression under anaerobic conditions. No equivalent to the AlcR regulator protein has, however, been found in any plant. Hence the *alcA/alcR* type of gene regulator system is not known in plants. Constitutive expression of AlcR in plant cells does not result in the activation of endogenous *adh* activity.

WO 93/21334 describes the production of transgenic plants which include such a system as a gene switch. This document specifically describes a chemically inducible plant gene

expression cassette comprising a first promoter operatively linked to a regulator sequence which encodes a regulator protein, and an inducible promoter operatively linked to target gene, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer, whereby application of the inducer causes expression of the target gene. Exogenous chemical inducers which are applied in this case include those described by Creaser *et al.*, J. Biochem. (1984) 225, 449-454) such as butan-2-one (ethyl methyl ketone), cyclohexanone, acetone, butan-2-ol, 3-oxobutyric acid, propan-2-ol and ethanol.

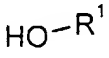
For agricultural purposes, alcohols are generally used as the exogenous chemical inducer. Other chemicals may, however, also be used a chemical inducers.

Our copending unpublished application PCT/GB99/04348 relates to the use of an agriculturally acceptable hydrolysable ester in the control of expression of a plant gene, said control being effected by an inducible promoter which requires for activation, the presence of an exogenous chemical which may comprise an alcohol, wherein hydrolysis of said agriculturally acceptable ester results in the production of said alcohol. Such an ester may be useful as a chemical inducer of the *alcA/ alcR* gene switch. In particular the agriculturally acceptable ester comprises a compound of formula (I)



(I)

in which R^1 is a lower alkyl, lower alkenyl or lower alkynyl group, and R^2 is a organic group such that R^2COOH is an agriculturally acceptable acid. Hydrolysis of a compound of formula (I) yields an alcohol of formula (II)



(II).

The term "agriculturally acceptable" as used herein means that the compounds may be applied to a particular soil or crop situation without causing unacceptable levels of soil damage or phytotoxicity in the crop.

The expression "lower alkyl" as used herein includes C_{1-6} alkyl groups, preferably from C_{1-4} alkyl groups which may be straight or branched chain. The expression "lower alkenyl" and "lower alkynyl" as used herein includes C_{2-6} alkenyl and C_{2-6} alkynyl groups respectively, preferably from C_{2-4} alkenyl or C_{2-4} alkynyl groups which may be straight or branched chain.

Agriculturally acceptable esters such as those of formula (I) are suitably translocated into the target plant in which the gene control system is in place and/or hydrolysed either under environmental conditions or in the presence of a suitable catalytic moiety such as an enzyme or catalytic antibody, at rates which are appropriate to provide sufficient quantities of the activating alcohol at the required time in the necessary parts of the plant. These may vary depending upon the nature of the plant species being treated, the gene being expressed and the timing of the application of the ester.

Esters such as the compounds of formula (I) are advantageous in that they are easier to handle than the corresponding alcohols. These compounds have been found to produce the desired effect in terms of gene activation.

The compound should be applied at a sufficient period of time prior to the required gene activation to allow hydrolysis to occur and this should be reasonable depending upon factors such as the growth stage of the plant at which activation is required. If the rate of hydrolysis is relatively slow, the time of application may be earlier in order to ensure that sufficient hydrolysis has occurred by the time the plant is at the growth stage at which gene activation is required. Where this is difficult, more rapidly hydrolysing esters may be selected.

Alternatively, more than one ester, with differing rates of hydrolysis may be applied in a single treatment. By selecting combinations of esters with different rates of hydrolysis, an effective "slow release" of activating alcohol can be achieved so that gene expression may be

prolonged over the desired period. This means that repeated applications of chemical may be avoided and "one-shot" treatments are possible.

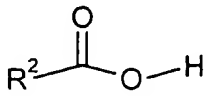
Particular examples of alcohols of formula (II) include methanol, ethanol, propan-1-ol, propan-2-ol, butan-2-ol or but-3-en-2-ol.

Suitably, the alcohol of formula (II) is a lower alkyl alcohol wherein the alkyl group has from 1 to 4 carbon atoms and may be either branched, or linear. Preferred groups for R^1 include ethyl, n-propyl and n-butyl.

A particularly preferred example of a compound of formula (II) is ethanol.

The precise nature of the R^2 group is immaterial provided that it gives rise to an agriculturally acceptable acid at an appropriate rate in the particular target plant to which it is applied. Rates of hydrolysis can be determined using routine methods for example as described by G. Mitchell et al., Pestic. Sci (1995) 44:49-58. and preferably by testing against whole plant systems. What is appropriate in any particular instance will depend upon a variety of factors including the nature of the gene expression of which is being controlled, the particular plant in which the gene is expressed and other external conditions. The rate of hydrolysis should be sufficient to allow the desired effect, for example, reversible male sterility, to be seen at an appropriate period of time after application of the chemical inducer.

R^2 however may be selected such that the resultant acid of formula (III)



(III)

has some useful agrochemical effect. In particular, it may itself be able to act as an inducer of the inducible promoter. For example, it has been found that a number of acids including 3-hydroxybutyric acid, 2-hydroxybutyric acid, pyruvic acid and 3-oxobutyric acid can act as an inducer of the alcR/alcA system (Creaser et al., supra.).

Particular examples of R^2 include optionally substituted alkyl, optionally substituted cycloalkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl or optionally substituted heterocyclyl.

As used herein the term "alkyl" includes straight or branched alkyl chains, suitably containing up to 10 carbon atoms, preferably from 1 to 6 carbon atoms. The terms "alkenyl" and "alkynyl" includes unsaturated straight or branched chains containing up to 10 carbon atoms, preferably from 2 to 6 carbon atoms. The term "aryl" includes phenyl and naphthyl. The term "heterocyclic" includes rings containing up to 10, preferably up to 7 atoms, up to three of which are selected from oxygen, sulphur or nitrogen. These rings may be single rings or may be in the form of fused ring systems and these may be aromatic or non-aromatic in nature. The term "halo" or "halogen" includes chlorine, fluorine, bromine and iodine. The term "alkoxy" relates to an alkyl group as defined above, linked with an oxygen atom.

Suitably R^2 is an optionally substituted C_{1-10} alkyl group which may be linear or branched. Preferred alkyl groups R^2 are linear and contain 3 to 8 carbon atoms, in particular 5 carbon atoms.

Suitable optional substitutents for alkyl, alkenyl and alkynyl groups R^2 include one or more groups selected from halo, nitro, cyano, oxo, optionally substituted aryl, optionally substituted heterocyclyl, OR^3, $C(O)_pR^3$, $S(O)_mR^3$, $OCOR^3$, $-NR^4C(O)_pR^3$, $=NOH$, NR^5R^6, $C(O)NR^5R^6$, $C(O)NR^3NR^5R^6$, $-CH=NOR^3$, $P(O)R^7R^8$ or $P(O)OR^7OR^8$, $NR^3CONR^5R^6$, $-N=CR^5R^6$, $S(O)_mNR^5R^6$ or $-NR^3S(O)_mR^4$, $-N=NR^3$ where each R^3, R^4, R^5, R^6, R^7 and R^8 are independently selected from hydrogen, alkyl, alkenyl, alkynyl, aryl or heterocyclyl , any of which may be optionally substituted by a functional group and in the case of aryl and heterocyclic groups, may also be substituted by alkyl, alkenyl or alkynyl groups; or R^5 and R^6 together with the atom to which they are attached, may additionally form, together with the atom to which they are attached, a ring which may be carbocyclic or heterocyclic; p is 1 or 2 and m is 0, 1, 2 or 3.

As used herein the term "functional group" refers to include halo, cyano, nitro, oxo, hydroxy, =NOR^{11}, C(O)$_p$R^{11}, OR^{11}, S(O)$_m$R^{11}, NR^{12}R^{13}, C(O)NR^{12}R^{13}, OC(O)NR^{12}R^{13}, -CH=NOR^{11}, -NR^{12}C(O)$_n$R^{11}, -NR^{11}CONR^{12}R^{13}, -N=CR^{12}R^{13}, S(O)$_m$NR^{12}R^{13} or -NR^{12}S(O)$_m$R^{11} where R^{11}, R^{12} and R^{13} are independently selected from hydrogen or optionally substituted hydrocarbyl, or R^{12} and R^{13} together form an optionally substituted ring which optionally contains further heteroatoms such as oxygen and nitrogen or S(O)R^{14}, where p is an integer of 1 or 2, m is 0 or an integer of 1-3 and R^{14} is hydrogen or alkyl.

Suitable optional substituents for hydrocarbyl groups R^{11}, R^{12} or R^{13} include halo, perhaloalkyl such as trifluoromethyl, mercapto, hydroxy, alkoxy, oxo, heteroaryloxy, alkenyloxy, alkynyloxy, alkoxyalkoxy, aryloxy (where the aryl group may be substituted by halo, nitro, or hydroxy), cyano, nitro, amino, mono- or di-alkyl amino, alkylamido or S(O)$_p$R^{14} where m and R^{14} are as defined above.

Examples of optional substituents on alkyl, alkenyl or alkynyl groups R^2 are one or more groups selected from oxo; alkoxycarbonyl in particular lower alkoxycarbonyl; cyano; halo such as chloro, fluoro or bromo; phenyl optionally subsituted with amino or mono-or dialkyl amino or alkyl such as methyl; OR^3 where R^3 is alkyl or heterocyclyl optionally substituted by halo or alkyl; S(O)$_m$R^{11} where m is 0 or 2 and R^{11} is alkyl or phenyl optionally substituted by alkyl; NR^{5}R^6 or C(O)NR^{5}R^6 where R^5 is hydrogen, methyl or methoxyethyl and R^6 is alkyl such as methyl, phenyl or benzyl optionally substituted with halo such as fluoro or chloro, alkyl such as methyl or trifluromethyl or alkoxycarbonyl where the alkyl moiety may carry a further alkoxycarbonyl group , or R^6 is heterocyclyl such as thiazinyl optionally substituted by alkyl and/or acetyl; -NR^{4}C(O)$_p$R^3 where p is 2, R^3 is alkyl and R^4 is alkyl optionally substituted with alkoxy carbonyl such as ethoxyl carbonyl alkyl; -NR^{3}S(O)$_m$R^4 where R^3 is hydrogen, R^4 is phenyl optionally substituted by halo such as chloro, and m is 2; C(O)NR^{3}NR^{5}R^6 where R^3 and R^5 are hydrogen and R^6 is phenyl optionally substituted by halo or alkoxy such as methoxy; S(O)$_m$NR^{5}R^6 where m is 2, R^5 is hydrogen and R^6 is alkyl optionally substituted by one or more alkoxycarbonyl groups; heterocylclyl such as furyl, pyridyl, pyridinyl or pyrazinyl, triazinyl, any or which may be optionally substituted by alkyl, halo, trihalomethyl, phenyl, halophenyl, cyano or oxo,

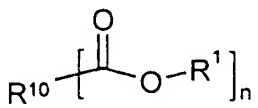
Particularly suitable substituents for alkyl, alkenyl or alkynyl groups R^2 include alkoxycarbonyl in particular where the alkoxy group is a lower alkyl group; alkoxy and in particular two alkoxy groups in the form of a dialkyl acetal; cyano or optionally substituted heterocyclyl. Preferred substituents include, but are not limited to lower alkoxycarbonyl groups and dialkyl acetals. Alkoxycarbonyl groups and dialkyl acetals are of particular interest when the alkyl group of the substituent is the same as R^1 in the compound of formula (I) since on hydrolysis these give rise to more inducer chemical of formula (II) .

A particular aryl group for R^2 is phenyl.

Suitable optional substituents for cycloalkyl, aryl and heterocyclyl groups R^2 and for aryl or heterocyclyl substituents on the above-mentioned alkyl, alkenyl or alkynyl groups R^2 include halo; haloalkyl; cyano; nitro; amino or mono- or di-alkyl amino; hydroxy; alkoxy, thioalkyl, alkyl or alkoxycarbonyl wherein the alkyl moiety of any of these may be optionally substituted with for example one or more groups selected from halo, alkoxy, cyano, alkoxycarbonyl, amino, mono- or di-alkyl amino, aryl or carboxylate or salts or esters thereof; cycloalkyl; or heterocyclyl.

Particularly suitable substituents for aryl or heterocyclyl groups R^2 include alkoxy in particular lower alkoxy such as methoxy, alkyl in particular lower alkyl, alkoxycarbonyl in particular lower alkoxycarbonyl and halogen.

A particular sub-group of compounds of formula (I) are compounds of formula (1A)



(IA)

where R^1 is as defined above in relation to formula (I), n is an integer of from 2 to 4 and R^{10} is an alkyl, alkenyl or alkynyl group any of which may be optionally interposed with a

heteroatom, a cycloalkyl, heterocyclic group or aryl group, or R^{10} is a cycloalkyl or aryl group of valency n.

In particular R^{10} is an alkyl or aryl group of valency n.

Particularly preferred compounds of formula (I) include:-

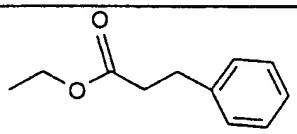
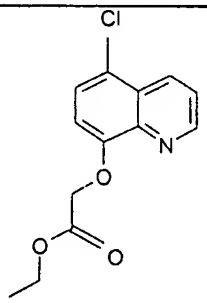
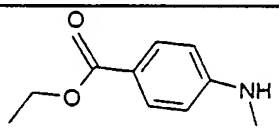
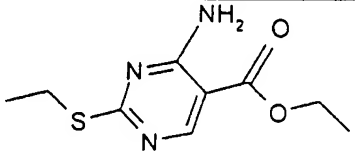
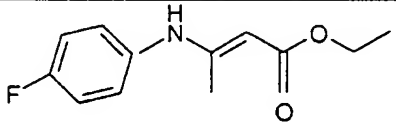
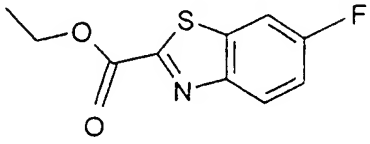
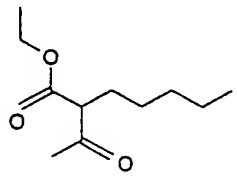
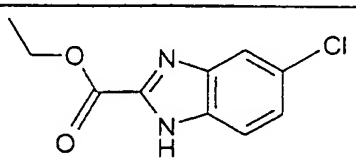
Ethyl 2-n-pentyl-3-oxobutanoate (Compound No. 49);

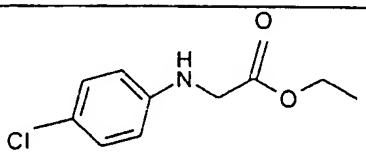
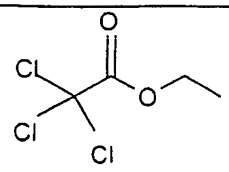
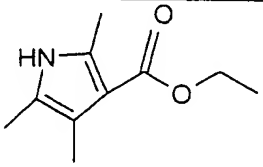
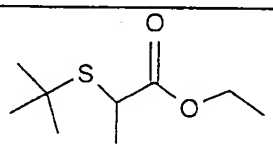
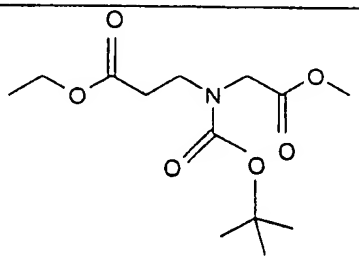
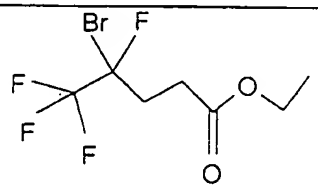
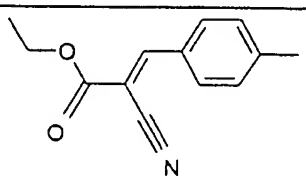
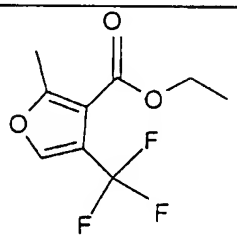
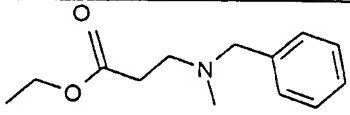
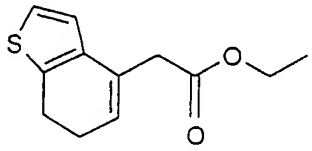
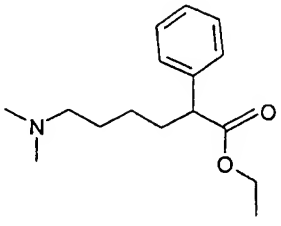
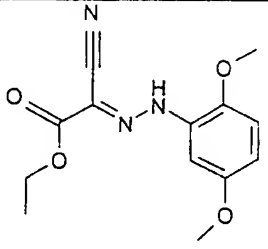
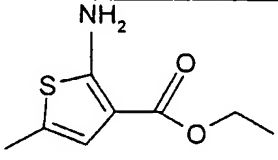
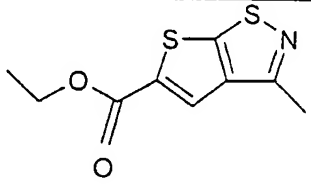
Triethyl 2-carboxyheptan-1,7-dioate (Compound No. 53); and

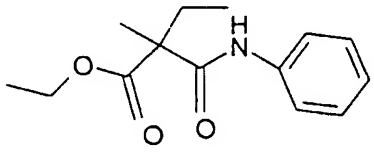
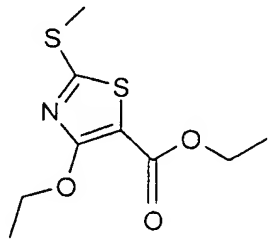
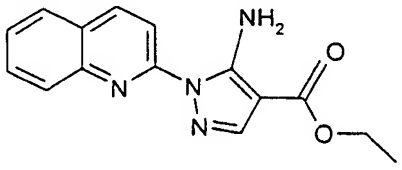
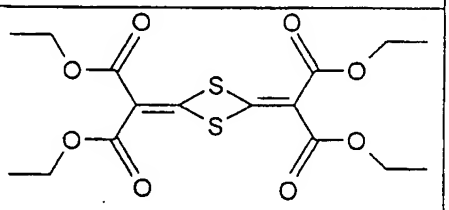
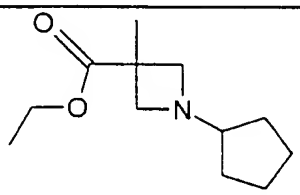
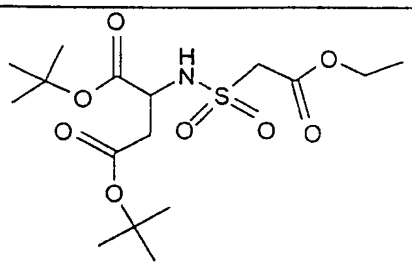
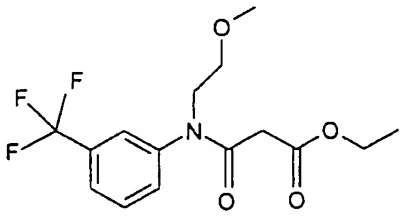
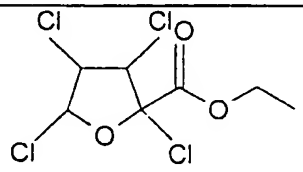
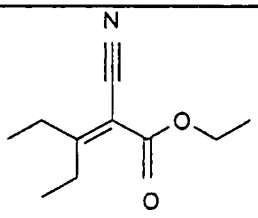
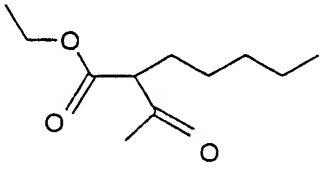
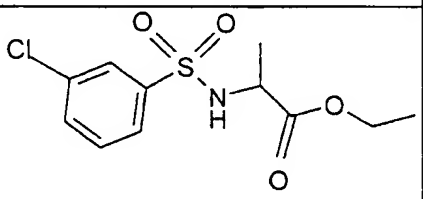
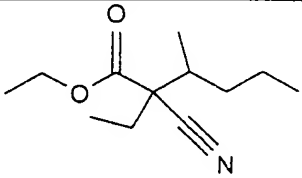
Ethyl 2,4-dimethoxybenzoate (Compound No. 60).

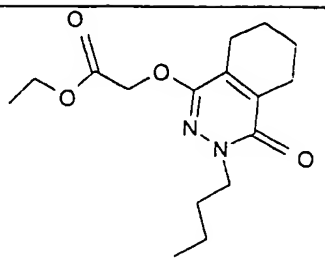
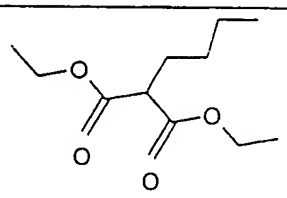
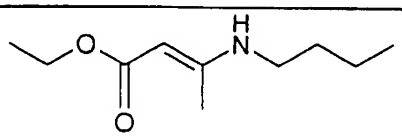
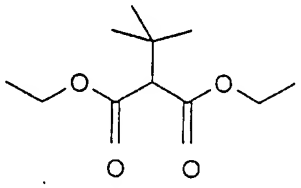
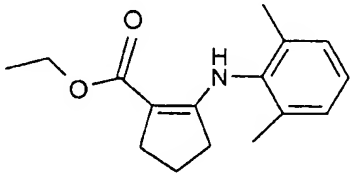
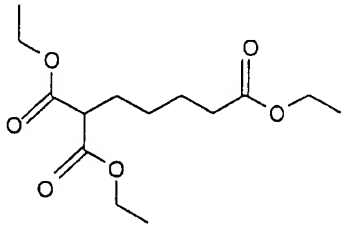
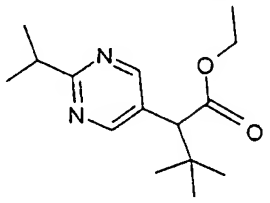
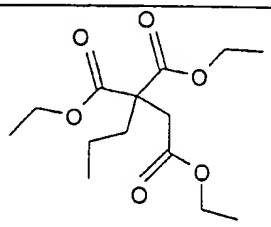
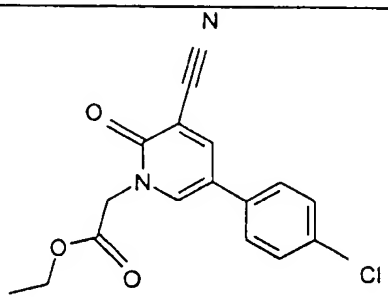
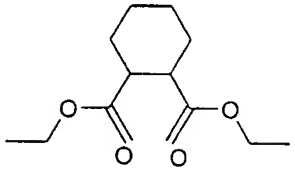
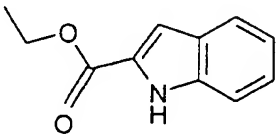
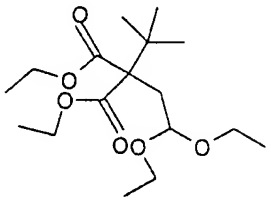
Examples of compounds of formula (I) described are ethyl esters as shown in Table 1.

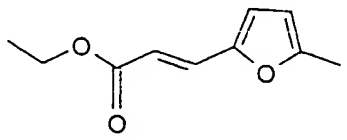
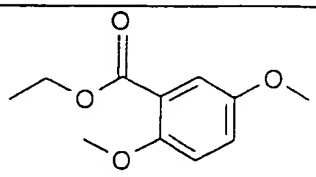
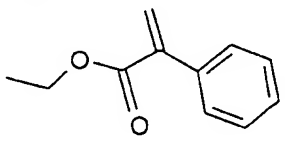
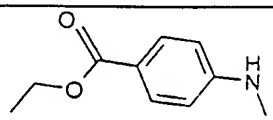
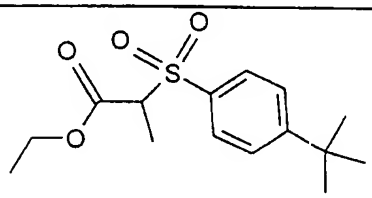
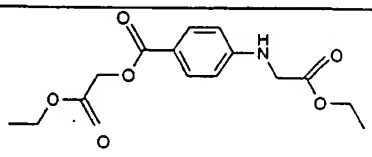
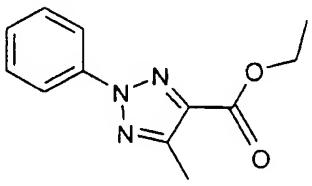
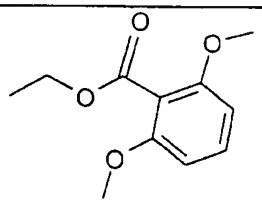
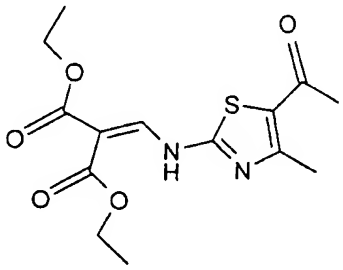
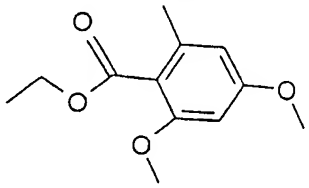
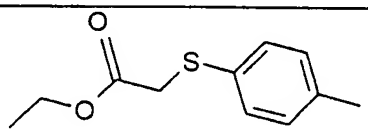
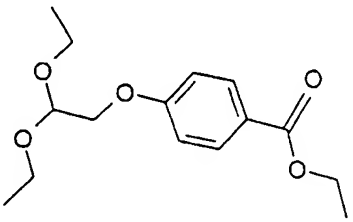
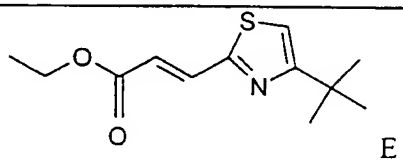
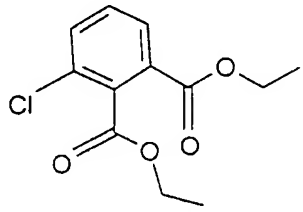
Table 1

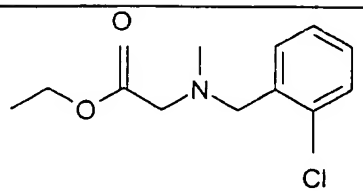
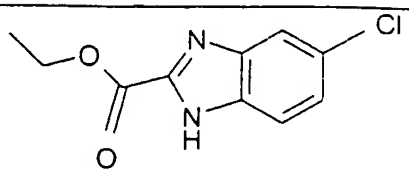
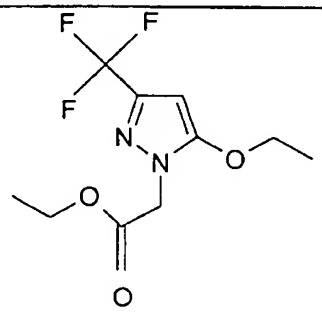
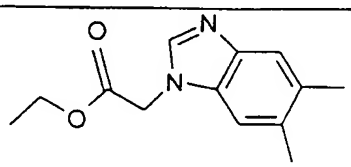
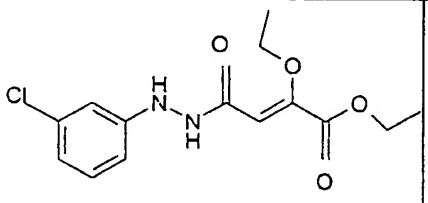
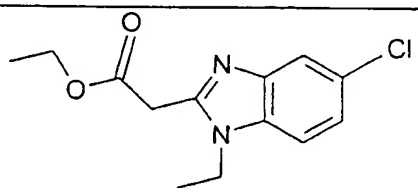
Compd. No.	Formula	Compd No.	Formula
1		34	
2		35	
3		36	
4		37	

5		38	
6		39	
7		40	
8		41	
9		42	
10		43	
11		44	

12		45	
13		46	
14		47	
15		48	
16		49	
17		50	

18		51	
19		52	
20		53	
21		54	
22		55	
23		56	

24		57	
25		58	
26		59	
27		60	
28		61	
29		62	
30	 E form	63	

31		64	
32		65	
33		66	

Compounds of formula (I) are either known compounds of they can be prepared from known compounds using conventional methods.

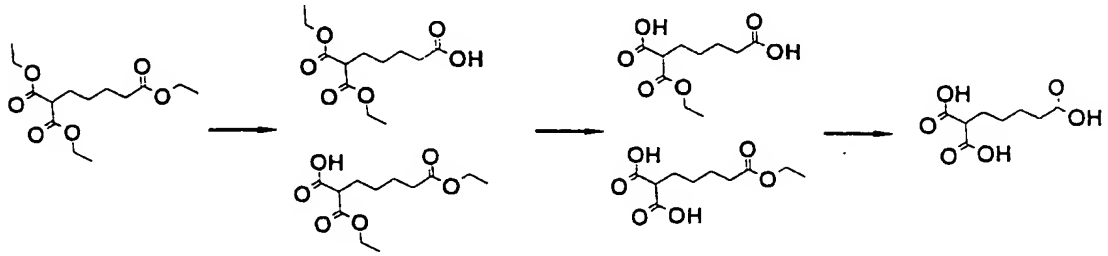
Compounds of formula (I) may be hydrolysed in the target plant either chemically, or enzymatically by a naturally occurring enzyme in the target plant or by an enzyme introduced by genetic engineering into the plant and expressed within the plant, or by an appropriate catalytic antibody, or catalytically active portion of a catalytic antibody introduced by genetic engineering into the plant and expressed within the plant.

Suitable enzymes include, but are not limited to, esterases and lipases.

Suitable catalytic antibodies may be generated by standard techniques from analogues of a tetrahedral ester hydrolysis transition state, e.g. as for the hydrolysis of the pro-drug ester of chloramphenicol, when appropriate phosphonates were used, Ole K et al., 1998, J. Mol.

Biol., 281:501-511. and for the detoxification of cocaine by methyl ester hydrolysis. Mets B et al., 1998. Proc. Nat. Acad. Sci. USA. 95:10176-10181.

An example of how it is believed a representative compound of the invention. Compound 53, is metabolised is shown in the following scheme:



Triester Mono Acids Di Acids Tri acid

A product of this metabolism is ethanol which can act as a chemical inducer as described above.

Another of our copending unpublished applications, PCT/GB99/04345, relates to the use of the use of a formulation comprising the components:

(a) a volatile chemical inducer;

(b) a polyethoxylated C_{10}-C_{20} alcohol or a trisiloxane polyethoxylate and

(c) a diluent;

for controlling expression of a target gene in an organism having a chemically-inducible gene expression cassette comprising an inducible promoter operatively linked to the target gene wherein the inducible promoter is induced by the application to the organism of (a) above. Suitably, the organism is a plant such as a crop plant and the diluent (c) may be, for example, water.

The nature of component (a) in the above formulations depends upon the character of the inducible promoter present in the expression system. However, particular examples of component (a) are a C_1-C_6 alcohol or a C_3-C_9 ketone, and preferably, ethanol or propan-2-ol. These components act as inducers for example, of the *alc* switch system as described above.

Component (b) of the formulation is preferably a polyethoxylated oleyl, lauryl, stearyl or cetyl alcohol. It is more preferably a polyoxyethylene-oleyl alcohol having a mean molar ethylene oxide content in the range of 0 to 35 and more preferably in the range of 2 to 20. It is most preferably a polyoxyethylene-(2)-oleyl alcohol, a polyoxyethylene-(10)-oleyl alcohol or a polyoxyethylene-(20)-oleyl alcohol. Component (b) is, however, preferably a polyoxyethylene-(20)-oleyl alcohol (the number in brackets indicates the mean ethylene oxide content per molecule). Such products are commercially available as BRIJ 92™, BRIJ 97™ and BRIJ 98™. Component (b) of the formulation is preferably at a concentration of about 0.5% wt/wt or less. It is preferably at a concentration between about 0.2% wt/wt and 0.5% wt/wt.

In an alternative embodiment, the formulation described includes as component (b), a hydrogen or methyl end-capped trisiloxane polyethoxylate In particular, component (b) is a methyl end-capped trisiloxane polyethoxylate. The methyl end-capped trisiloxane polyethoxylate preferably has a mean molar ethylene oxide content of between 4 and 12 per molecule and is most preferably 8 per molecule. Such products are commercially available as SILWET 77™ (SILWET is a trademark of Witco). The methyl end-capped trisiloxane polyethoxylate is preferably at a concentration of about 0.5% wt/wt or less and more preferably at a concentration between about 0.2% and 0.5% wt/wt.

Component (a) of the formulation described is preferably at a concentration of about 5% wt/wt or less and more preferably at a concentration between about 2% and 5% wt/wt.

Component (c) of the formulation is preferably at a concentration between about 90% and 98% wt/wt.

Also described is an agricultural formulation, comprising

(a) a C_1-C_6 alcohol inducer of an inducible promoter in an amount of less than 5%wt/wt;

(b) a polyethoxylated C_{10}-C_{20} alcohol; and

(c) water.

In this case, component (b) of the formulation is suitably a polyethoxylated oleyl, lauryl, stearyl or cetyl alcohol, and preferably a polyoxyethylene-oleyl alcohol. Suitably, the polyoxyethylene-oleyl alcohol has a mean molar ethylene oxide content in the range of 2 to 20, such as a polyoxyethylene-(2)-oleyl alcohol, a polyoxyethylene-(10)-oleyl alcohol or a polyoxyethylene-(20)-oleyl alcohol. Again component (a), which is preferably ethanol or propan-2-ol, is suitably at a concentration between about 2% to less than 5% wt/wt. The concentration of component (B) is preferably about 0.5% wt/wt or less.

A further agricultural formulation is described comprising

(a) a C_3-C_9 ketone which is able to act as a chemical inducer of an inducible promoter;

(b) a polyethoxylated C_{10}-C_{20} alcohol; and

(c) a diluent.

Component (a) is preferably

at a concentration between about 2% and 5% wt/wt. As before, component (b) may be a polyethoxylated oleyl, lauryl, stearyl or cetyl alcohol, and is preferably a polyoxyethylene-oleyl alcohol, with a mean molar ethylene oxide content in the range of 2 to 20. Particular examples of component (b) are polyoxyethylene-(2)-oleyl alcohol, polyoxyethylene-(10)-oleyl alcohol and polyoxyethylene-(20)-oleyl alcohol.

The formulations increase the effectiveness of ethanol as an inducer of the *alcA/alcR* promoter. In addition, the use of polyoxyethylene-oleyl alcohol surfactants and/or methyl end-capped trisiloxane polyethoxylate adjuvants in combination with the known chemical inducers for the *alcA/alcR* promoter has been found to increase uptake of the formulation by a plant significantly, thereby allowing greater control of a target gene which may be operatively linked to the inducible promoter.

It is desirable to use a plant gene expression system which provides higher levels of inducible expression in transgenic plants and which, preferably, also provides a higher proportion of high expressing plant lines.

The present invention therefore seeks to provide an improved plant gene expression system. In particular, the present invention seeks to provide a chemically-inducible promoter based on the *alcA/ alcR* promoter system.

According to a first aspect of the present invention, there is provided a chemically- inducible plant gene expression cassette comprising a first promoter operatively linked to the *alcR* regulator protein sequence obtainable from *Aspergillus nidulans* which encodes the AlcR regulator protein, and an inducible promoter obtainable from the *alcA* gene promoter of *Aspergillus nidulans* operatively linked to a target gene, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene and wherein the *alcA* promoter has three or more binding sites for the AlcR protein.

The exogenous inducer of plant gene expression is preferably an alcohol, ketone, a hydrolysable ester or a formulation comprising a volatile chemical inducer, a polyoxyethylated C_{10}-C_{20} alcohol or a trisiloxane polyoxylate and a diluent.

Preferably, the inducible promoter of the plant gene expression cassette is a chimaeric promoter and preferably comprises the polynucleotide sequence of SEQ. ID. NO. 3 or at least part of a sequence that has substantial identity therewith or a variant or fragment thereof.

Even more preferably, the inducible promoter sequence of the plant gene expression cassette is a non-chimaeric promoter. In this case, the inducible promoter of the plant gene expression cassette preferably comprises the polynucleotide sequence of SEQ. ID. NO. 2 or the polynucleotide sequence of SEQ. ID. NO. 4 or at least part of a sequence that has substantial identity therewith or a variant or fragment thereof.

According to a second aspect of the present invention, there is provided an expression system comprising a gene expression cassette as defined above.

According to a third aspect of the present invention, there is provided a plant cell comprising a gene expression cassette as defined above. Preferably, the gene expression cassette is incorporated, preferably stably incorporated, into the plant's genome.

According to a fourth aspect of the present invention, there is provided plant tissue comprising a plant cell according to the present invention.

According to a fifth aspect of the present invention, there is provided a plant comprising a plant cell according to the present invention.

According to a sixth aspect of the present invention, there is provided a plant obtainable from a plant as defined above.

According to a seventh aspect of the present invention, there is provided a seed obtainable from a plant as defined above.

According to an eighth aspect of the present invention, there is provided a method for controlling plant gene expression comprising transforming a plant cell with a chemically inducible plant gene expression cassette comprising a first promoter operatively linked to the *alcR* regulator protein sequence obtainable from *Aspergillus nidulans* which encodes the AlcR regulator protein, and an inducible promoter obtainable from the *alcA* gene promoter of *Aspergillus nidulans* operatively linked to a target gene, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene and wherein the *alcA* promoter has three or more binding sites for the AlcR protein.

According to a nineth aspect of the present invention, there is provided a chemically inducible promoter comprising an upstream region comprising a promoter regulatory

sequence obtainable from the *alcA* gene promoter of *Aspergillus nidulans* and a downstream region comprising a transcription initiation sequence, wherein the *alcA* gene promoter sequence comprises three or more binding sites for the AlcR regulatory protein.

Preferably, the promoter is a chimaeric promoter. It preferably comprises the polynucleotide sequence of SEQ. ID.NO 3 or at least part of a sequence that has substantial identity therewith or a variant or fragment thereof.

Alternatively, the promoter may be a non-chimaeric promoter. It preferably comprises the polynucleotide sequence of SEQ. ID.NO 2 or at least part of a sequence that has substantial identity therewith or a variant or fragment thereof.

Preferably, the promoter comprises 4 direct repeat binding sites for the AlcR protein. It preferably comprises the polynucleotide sequence of SEQ.ID.NO 4 or at least part of a sequence that has substantial identity therewith or a variant or fragment thereof.

According to a tenth aspect of the present invention there is provided the use of a polynucleotide sequence as defined above in the production of other chemically-inducible promoter variants using molecular evolution and/ or DNA shuffling methods.

According to a preferred embodiment of the present invention, there is provided a chemically- inducible plant gene expression cassette comprising a first promoter operatively linked to the *alcR* regulator protein sequence obtainable from *Aspergillus nidulans* which encodes the AlcR regulator protein, and an inducible promoter obtainable from the *alcA* gene promoter of *Aspergillus nidulans* operatively linked to a target gene, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene, wherein the *alcA* promoter has three or more binding sites for the AlcR protein and wherein the inducible promoter is a non-chimaeric promoter comprising 4 direct repeat binding sites for the AlcR protein.

According to an even more preferred embodiment of the present invention, there is provided a chemically-inducible promoter comprising an upstream region comprising a promoter regulatory sequence obtainable from the *alcA* gene promoter of *Aspergillus nidulans* comprising three or more binding sites for the AlcR protein, wherein the promoter is a non-chimaeric promoter comprising 4 direct repeat binding sites for the AlcR protein.

The term " target gene" with reference to the present invention means any gene of interest (GOI). Such a gene may be an endogenous plant gene or a foreign gene, and may be a single gene or a series of genes. The target gene sequence may encode at least part of a functional protein or an antisense sequence.

Typical examples of a GOI include a gene which gives rise to the desired characteristic or phenotype itself. The inducible promoter may control expression of a repressor protein which inhibits expression of a target gene, for example by interacting with an operator sequence upstream of the target gene so as to prevent expression of the gene (for example as known in the bacterial *tet* and *lac* operator/repressor systems). In a further alternative, the GOI under the control of the inducible promoter may express a protein which interacts with another protein to inhibit the activity thereof, as for example in the barnase/ barstar system which barnase will inhibit or kill cells in the absence of barstar. Other disrupter genes include ribonuclease, β-tubulin or adenine nucleotide translocator (ANT).

The term "substantial identity" covers identity with respect to at least the essential nucleic acids/ nucleic acid residues of the promoter sequence providing the homologous sequence acts as a chemically inducible promoter which is capable of inducing a GOI. A preferred level of identity is shown when 60% or more of the nucleotides are common with the promoter sequence of the present invention, more preferably 65%, 70% or 75%, even more preferably 80% or 85% and, especially preferred are 90%, 95%m 98% or 99% or more levels of identity. When comparing nucleic acid sequences for the purposes of determining the degree of identity one can use programs such as BESTFIT and GAP (both from Wisconsin Genetics Computer Group (GCG) software package). BESTFIT, for example, compares two sequences and produces an optimal alignment of the most similar segments. GAP enables

sequences to be aligned along their whole length and finds the optimal alignment by inserting spaces in either sequence as appropriate. Suitably, in the context of the present invention, the comparison is made by alignment of the sequences along their whole length.

The term "variant thereof" with reference to the present invention means any substitution of variation of , modification of , replacement of, deletion of or the addition of one or more nucleic acid(s) from or to the promoter sequence providing the resultant sequence exhibits inducible promoter of a particular GOI. The term also includes DNA which substantially hybridises to the DNA of the present invention and which functions as a chemically inducible promoter. Preferably, such hybridisation occurs at, or between, low and high stringency conditions. In general terms, low stringency conditions can be defined as 3 x SSC at about ambient temperature to about 65 °C, and high stringency conditions as 0.1 x SSC at about 65 °C. SSC is the name of the buffer of 0.15M NaCl, 0.015M trisodium citrate. 3 x SSC is three times as strong as SSC and so on.

The sequences according to the present invention may also be used in the production of other variants using molecular evolution and/ or DNA shuffling techniques. Such techniques are described, for example, in US Patent No. 5 605 793, US Patent No. 5 811 238 and US Patent No. 5 830 721. In essence, this technique involves expression of a gene (or nucleic acid sequence) in a microbial expression system such as *Escherichia coli*. The particular system selected must be validated and calibrated to ensure that biologically active peptides are expressed, which may be readily achieved using an *in vivo* bioassay. The gene, or preferably a collection of related genes from different species, may be subject to mutagenic polymerase chain reaction (PCR) as is known in the art. Fragmentation of the products and subsequent repair using PCR leads to a series of chimaeric sequences reconstructed from parental variants. These chimaeras are then expressed in the microbial system which can be screened in the usual way to determine active mutants, which may then be isolated and sequenced. Reiteration of this molecular evolution DNA shuffling cycle may lead to progressive enhancement of the desired properties. The advantage of a technique of this nature is that it allows a wide range of different mutations, including multi-mutational block exchanges, to be produced and screened.

Particular variants are those derivable from polynucleotides of SEQ. ID. Nos 2, 3 and/ or 4. Preferably such variants will have improved chemical inducibility compared to known promoter sequences.

As used herein, the term "fragment thereof" includes one or more regions of the basic sequence which retain promoter activity. When the fragments comprise one or more regions, they may be joined together or they may be spaced apart by additional bases.

The term "DNA construct" - which is synonymous with terms such as "cassette", "hybrid", "conjugate" and "vector" - includes a target gene directly or indirectly attached to the regulator promoter, such as to form a cassette. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence intermediate the promoter and the target gene. The DNA sequences may, furthermore, be on different vectors and are therefore not necessarily located on the same vector. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. Such constructs also include plasmids and phage which are suitable for transforming a cell of interest.

The term "expression system" means that the system defined above can be expressed in an appropriate organism, tissue, cell or medium. The system may comprise one or more constructs and may also comprise additional components that ensure the increased expression of the target gene.

Any transformation method suitable for the target plant or plant cells may be employed, including infection by *Agrobacterium tumefaciens* containing recombinant Ti plasmids, electroporation, microinjection of cells and protoplasts, microprojectile bombardment, bacterial bombardment, particularly the "fibre" or "whisker" method, and pollen tube trnasformation. The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the genome. Both

transformed monocot and dicot plants may be obtained in this way. Reference may be made to the literature for full details of the known methods.

Examples of genetically modified plants which may be produced include dicotyledenous and moncotyledonous plant such as field crops, cereals, fruits and vegetables such as : canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strwberries, bananas, melons, potatoes, carrot, lettuce, cabbage and onion.

An advantage of the chemically-inducible promoter of the present invention is that it has increased and greater uniformity in inducible expression levels over known inducible promoters. The chemically-inducible promoter of the present invention, therefore, has enhanced effects over known inducible promoters in areas such as in agriculture.

A further advantage of the plant gene expression system is that it provides higher levels of inducible expression in transgenic plants and that it also provides a higher proportion of high expressing lines in the primary transformant plant population.

Another advantage of the present invention is the the *alc A* promoter may be enhanced by the addition of extra *alcR* binding sites and/ or the removal of the 35S minimal promoter.

The *alcA* promoter described in International Publication No. WO 93/21334 comprises a -33 base pair 35S minimal promoter sequence at the 3' end, which replaces the native *alcA* promoter sequence in this region. The promoter was made chimaeric since it was believed that expression of the fungal promoter would be enhanced in a plant system. This version, pSRN1ACN1, has been demonstrated to be a good inducible promoter system in several plant species.

Three altered *alc A* constructs have been made.

The first, pSRN1ACN2, was made with the native *alcA* sequence replacing the 35S sequence in this region and has three binding sites for the AlcR regulatory protein.

The second, pSRN1ACN3, was made with almost the full native *alcA* promoter sequence and has three binding sites for the AlcR regulatory protein. This construct also comprises a -33 base pair 35S minimal promoter.

The third version of the *alcA* construct, pSRN1ACN8, comprises 100 bases of the native *alcA* sequence at the 3' end of the promoter and four inverted repeats of the *alcR* binding sites placed upstream of this.

pSRN1ACN1 represents the original *alcA* promoter which has only two *alcR* binding sites.

The results show that the inducible expression levels of the plants which contained the *alcA* promoter without the minimal 35S sequence (pACN2) were higher than in the same *alcA* promoter sequence with the minimal 35S sequence (pACN3). In addition, there was a greater number of expressing lines in the pACN2 population of transgenic plants. This suggests that the presence of the 35S minimal promoter may be detrimental to obtaining consistent high expression in the primary transgenic plant lines.

The comparison of a tobacco primary transgenic population with the original *alcA* promoter (pACN1) which has two *alcR* binding sites to the *alcA* promoter (pACN3) with three *alcR* binding sites shows that there is a higher level of inducible expression among the primary transformant plants when there is an extra *alcR* binding site present.

The results of the analysis of the plants containing an *alcA* promoter consisting of four *alcR* inverted repeat binding sites and 100 base pairs of the 3' end of the *alcA* promoter (pACN8) show that this is sufficient to allow inducible expression to be greatly enhanced when compared to the known version of the *alcA* promoter (pACN1 shown in Figure 1).

The present invention will now be described only by way of non-limiting example with reference to the accompanying drawings in which:-

Figure 1 shows the vector constructs for pACN1, pACN2, pACN3 and pACN8;

Figure 2 shows the nucleic acid sequences. SEQ. ID. Nos 1-4, for the inducible promoter in each of the vector constructs;

Figure 3 shows the results showing expression levels of primary transgenic tobacco plants with different ALC optimisation constructs, SRN1ACN1, SRN1ACN2, SRN1ACN8 and SRN1ACN1;

Figure 4 shows a comparison of CAT protein levels in ALCCAT primary tranformant populations 72 hours after induction *including* those showing zero activity;

Figure 5 shows a comparison of CAT protein levels in ALCCAT primary transformant populations 72 hours after induction *excluding* those showing zero activity;

Figure 6 shows the pCa MVCN vector; and

Figure 7 shows the pAN204 vector.

EXAMPLE 1

Design of constructs

Four versions of the ALC switch constructs were transformed into tobacco. These constructs comprised a cassette with an *alcR* gene under the control of a 35S promoter and nos terminator (described hereafter as SRN), and a CAT reporter gene under the control of an *alcA* inducible promoter and with a nos terminator (described hereafter as ACN). However, the *alcA* promoter in the ACN cassette had been altered in three of the constructs as described below (see Figures 1 and 2).

1. The plasmid SRN1ACN1 is the same as described in WO 93/21334. It has the minimal 35S sequence in the *alc A* promoter as described and comprises two *alcR* binding sites.

2. The plasmid SRN1ACN2 differs from SRN1ACN1 in that the native *alcA* promoter is used which contains no minimal 35S sequence. It also contains more of the *alcA* sequence at the 5' end. This plasmid comprises three *alcR* binding sites.

3. The plasmid SRN1ACN3 differs from SRN1ACN2 in that it contains the minimal 35S sequence in the *alcA* promoter in place of the *al A* sequence at the 5' end.

4. The plasmid SRN1ACN8 contains 98 bases of the 3' end of the *alcA* promoter of SRN1ACN1, and in addition there are 4 direct repeat *alcR* binding sites of sequence GTCCGCACGGGATGTCCGCACGG located at the 5' end of this *alcA* promoter region.

EXAMPLE 2

Cloning details

pACN1 (in SRN1ACN1)

1. A pCaMVCN vector (Figure 6) with the 35S promoter, *E coli* CAT reporter gene and nos terminator was obtained. The HindIII site 3' to the nos terminator was removed by blunt ending and religation. The plasmid was then cut with BglII and ClaI and the resulting recessed nucleotides filled in with Klenow DNA polymerase. The fragments were blunt end ligated to form pCaMVCNΔH.

2. The *alcA* promoter was fused to the CaMV35S promoter minimal sequence using recombinant PCR technology, utilising an identical core sequence of TCTATATAA in the TATA boxes of the two promoters. In this way, the sequence 3' to this in the *alcA* promoter was replaced with 32 bases of CaMV35S sequence.

   - A fragment of approximately 246bp was amplified from a pAN204 plasmid (Figure 7) containing the ALC A promoter using the following oligonucleotides

A1 5' GCAAGCTTCGGGATAGTTCCGACC

A2 5' gaacttccttatatagatgttcagc

- A fragment of approximately 300bp was amplified from the pCaMVCN vector using the following oligonucleotides

C2 5' tctatataaggaagttc

C3 5' TCACCAGCTCACCGTCT

- The two PCR products were purified and amplified by recombinant PCR with A1 and C1 oligonucleotides to produce a fragment of approximately 600bp.

C1 5' ACGGAATTCCGGATGAG

The fused fragment contained a 5' HindIII site and a BamHI site near the 3' end, and these enzymes were used to cut out the recombinant *alcA* promoter sequence. This was cloned into the pCaMVCNΔH plasmid digested with HindIII and BamHI enzymes. The HindIII site 3' to the nos terminator sequence was restored by removal of the 1000bp EcoR1 fragment and replacing it with the same fragment of pCaMVCN, to give pACN1.

pACN2 (in SRN1ACN2)

1. The native *alcA* promoter was amplified by PCR from the clone pAN204, adding HindIII, EcoRI and PacI to the 5' end and BamHI, NotI to the 3' end.

2. The plasmid pACN1 was digested with HindIII (partial digest) and BamHI, which removed the *alcA/* 35S minimal sequence promoter. The fragment left was the vector with the CAT gene and nos terminator.

3. The PCR product of the native *alcA* promoter was digested with HindIII and BamHI, and ligated into the vector with the CAT gene and nos terminator from (2) above.

pACN3 (in pSRNACN3)

1. The native *alcA promoter* was amplified by PCR from the genomic clone pAN204 (Lockington et al, Gene 33 (1985) 137-149) adding HindIII; EcoRI, PacI to the 5' end and BamHI, NotI to the 3' end.

2. Similarly the *alcA*/minimal 35S chimeric promoter was amplified by PCR from pACN1, adding EcoRI, HindIII to the 5' end and PstI, SalI BamHI and NotI to the 3' end.

3. In both fragments there is an ApaI site approximately 100 base pairs from the 3' end. Both PCR products were digested with ApaI and religated so that digestion fragments from the two PCR products could ligate. The ligation product with the 5' end from pAN204 (native *alcA*) and the 3' end from pACN1 (with the minimal 35S promoter) was obtained. This is pACN3. The fusion promoter was checked by sequencing.

pACN8 (inSRN1ACN8)

Oligonucleotides based on the -140 bp direct repeat *alcR* binding site in the native *alcA* promoter were synthesised and annealed to form a linker.

XhoI KpnI SalI			
TCGAGTCCGCACGGGATGTCCGCACGGGGTACCG	3'	oligoalcRA	
CAGGCGTGCCCTACAGGCGTGCCCCATGGCAGCT	5'	oligoalcRB	

This was phosphorylated and mutimerised by ligation. As the ends of XhoI and SalI are compatible, this ligation mix contained a mixture of XhoI/SalI; XhoI/XhoI; and SalI/SalI ligated ends. The fragments with the SalI/XhoI ligated ends are required to form multimers of the direct repeat. The ligation products are digested with XhoI and SalI to digest all the XhoI/XhoI and SalI/SalI joins, but do not cut the required SalI/XhoI ends. The digests are run on a 2.5% agarose gel and bands of visible sizes are extracted. These are recovered and separately ligated into the XhoI site of a plasmid which has a XhoI site 5' to a 98bp minimal native *alcA* promoter sequence.

The 4 constructs were transformed into tobacco. The plants were transformed according to the method of Bevan (Nucleic Acids Res 1984 Nov 26;12(22):8711-8721). 3-4 week old sterile culture of tobacco (*Nicotiana tabacum* cv Samsum), grown on MS, were used for the transformation. The edges of the leaves were cut off and the leaves cut into pieces. They were then incubated in a suspension of *Agrobacterium* LBA4404 containing the binary vector, for 20 min. They were then put on plates containing NBM media (MS supplemented with 1mg/litre 6-benzylamino purine (6-BAP), 0.1mg/litre napthalene acetic acid (NAA). After 2 days explants were transferred to culture pots containing the NBM medium supplemented with carbenicillin (500mg/L) and kanamycin (100mg/L). Five weeks later, 1 shoot per leaf disc was transferred to NBM medium supplemented with carbenicillin (200mg/L) and kanamycin (100mg/L). After 2-3 weeks, shoots that had rooted were transferred to fresh medium

EXAMPLE 3

Plant Analysis

In order to identify transgenic primary transformants, leaf samples were taken from all the kanamycin resistant plants in tissue culture and extracts made for PCR analysis. The oligonucleotides 35Sjp1 and AlcRjp4R were used to amplify extracts from all four populations. The PCR conditions were 94°C, 30 seconds; 60°C, 30 seconds and 72°C, 60 seconds, for 30 cycles.

35Sjp1 5' cgt tga aga tgc ctc tgc cga c

AlcRjp4R 5' agt ctg ctc ttc cag ctc gcg

In addition, the oligonucleotides alcRjp2 and catjp2R were used to screen the SRN1ACN1 population. The PCR conditions were 94°C, 45 seconds; 60°C, 45 seconds and 72°C, 90 seconds, for 30cycles.

AlcRjp2 5' ctg gac tgt tta gat ctg ga

catjp2R 5' acc cag gga ttg gct gag acg

The primary transgenic plants were potted into 3 inch pots in soil and left to grow until approximately 14 cms high, 6-8 expanded leaf stage. Leaf samples were taken from the uninduced plants, frozen in a dry ice ethanol bath and stored at -70°C. The plants were then root drenched with 5% ethanol. After 3 days, leaf samples were taken. These samples and the uninduced samples stored at -70°C were ground in 200ul of 250mM Tris buffer pH 8.0, centrifuged and the supernatant recovered and stored at 4^0C overnight. The samples were analysed for CAT protein quantification by using a Boehringer Mannheim CAT ELISA kit and the total protein level determined by a Bradford determination.

EXAMPLE 4

Results

The results are shown in Figure 3, and show that the transgenic plants with the ACN1, ACN2, ACN3 and ACN8 reporter cassettes are inducible with ethanol.

1. The percentage of plants expressing CAT protein after ethanol induction is higher in the SRN1ACN2 and SRN1ACN8 populations than in the SRN1ACN1 population.

2. The number of transgenic plants with high expression levels after induction with ethanol (10-30ngCAT/mg total protein) is higher in the SRN1ACN2 population than in the SRN1ACN1 population.

It can be seen from Figures 4 and 5 that constructs ACN2, 3 and 8 show more consistent expression levels than the original ACN1 construct i.e. the spread of values is more even and the mean corresponds more closely to the median in ACN2, 3 and 8.

The results clearly show that the *alcA* promoter functions well as an inducible promoter in plants both with and without the minimal 35S sequence. Both the native (no minimal 35S promoter) and deleted *alcA* promoter fragment of 98 bases with *alcR* binding sites added at

the 5` end show enhanced inducibility. The minimal 35S promoter is not required in the *alcA* promoter for expression of the *alcA/ alcR* inducible system in plants.

EXAMPLE 5

In order to analyse whether there is less variation in expression levels with the homozygous or with the heterozygous plants of pSRNACN1 to pSRNACN2, a comparison of expression in segregating populations is carried out. A finding of less variation in induced expression of pSRNACN2 supports the suggestion that the two 35S components in pSRNACN1 may have a silencing effect.

Other modifications to the present invention will be apparent to those skilled in the art without departing from the scope of the present invention.

CLAIMS

1. A chemically inducible plant gene expression cassette comprising a first promoter operatively linked to the *alcR* regulator protein sequence obtainable from *Aspergillus nidulans* which encodes the AlcR regulator protein, and an inducible promoter operatively linked to a target gene, the inducible promoter obtainable from the *alcA* gene promoter of *Aspergillus nidulans* being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene, the *alcA* promoter having three or more binding sites for the AlcR protein.

2. A plant gene expression cassette according to claim 1 wherein the exogenous inducer is an alcohol, ketone, a hydrolysable ester or a formulation comprising a volatile chemical inducer, a polyoxyethylated C_{10}-C_{20} alcohol or a trisiloxane polyoxylate and a diluent.

3. A plant gene expression cassette according to claim 1 or claim 2 wherein the inducible promoter is a chimaeric promoter.

4. A plant gene expression cassette according to any one of claims 1 to 3 wherein the inducible promoter comprises the polynucleotide sequence of SEQ. ID. NO. 3 or at least part of a sequence that has substantial identity therewith or a variant or fragment thereof.

5. A plant gene expression cassette according to claim 1 or claim 2 wherein the inducible promoter sequence is a non-chimaeric promoter.

6. A plant gene expression cassette according to claim 5 wherein the inducible promoter comprises the polynucleotide sequence of SEQ. ID. NO. 2 or the polynucleotide sequence of SEQ. ID. NO. 4 or at least part of a sequence that has substantial identity therewith or a variant or fragment thereof.

7. An expression system comprising a gene expression cassette according to any one of the preceding claims.

8. A plant cell comprising an expression system according to claim 7.

9. A plant cell according to claim 8 wherein the expression system is incorporated, preferably stably incorporated, into the plant's genome.

10. A plant tissue comprising a plant cell according to claim 8 or claim 9.

11. A plant comprising a plant cell according to claim 8 or claim 9.

12. A plant obtainable from a plant according to claim 11.

13. A seed obtainable from a plant according to claim 11 or claim 12.

14. A method for controlling plant gene expression comprising transforming a plant cell with a chemically inducible plant gene expression cassette comprising a first promoter operatively linked to the *alcR* regulator protein sequence obtainable from *Aspergillus nidulans* which encodes the AlcR regulator protein, and an inducible promoter operatively linked to a target gene, the inducible promoter obtainable from the *alcA* gene promoter of *Aspergillus nidulans* being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene, the *alcA* promoter having three or more binding sites for the AlcR protein.

15. A chemically inducible promoter comprising an upstream region comprising a promoter regulatory sequence obtainable from the *alcA* gene promoter of *Aspergillus nidulans* and a downstream region comprising a transcription initiation sequence, wherein the *alcA* promoter sequence comprises three or more binding sites for the AlcR regulatory protein..

16. A promoter according to claim 15 wherein the promoter is a chimaeric promoter.

17. A promoter according to claim 16 comprising the polynucleotide sequence of SEQ. ID.NO 3 or at least part of a sequence that has substantial identity therewith or a variant or fragment thereof.

18. A promoter according to claim 15 wherein the promoter is a non-chimaeric promoter.

19. A promoter according to claim 18 comprising the polynucleotide sequence of SEQ. ID.NO 2 or at least part of a sequence that has substantial identity therewith or a variant or fragment thereof.

20. A promoter according to claim 18 wherein the promoter additionally comprises 4 direct repeat binding sites for the AlcR protein.

21. A promoter according to claim 20 comprising the polynucleotide sequence of SEQ. ID.NO 4 or at least part of a sequence that has substantial identity therewith or a variant or fragment thereof.

22. The use of a polynucleotide sequence according to any one of claims 15 to 21 in the production of other chemically-inducible promoter variants using molecular evolution and/ or DNA shuffling methods.

23. An expression cassette, a plant, a method or a promoter substantially as hereinbefore described with reference to the Figures.

Fig.1.

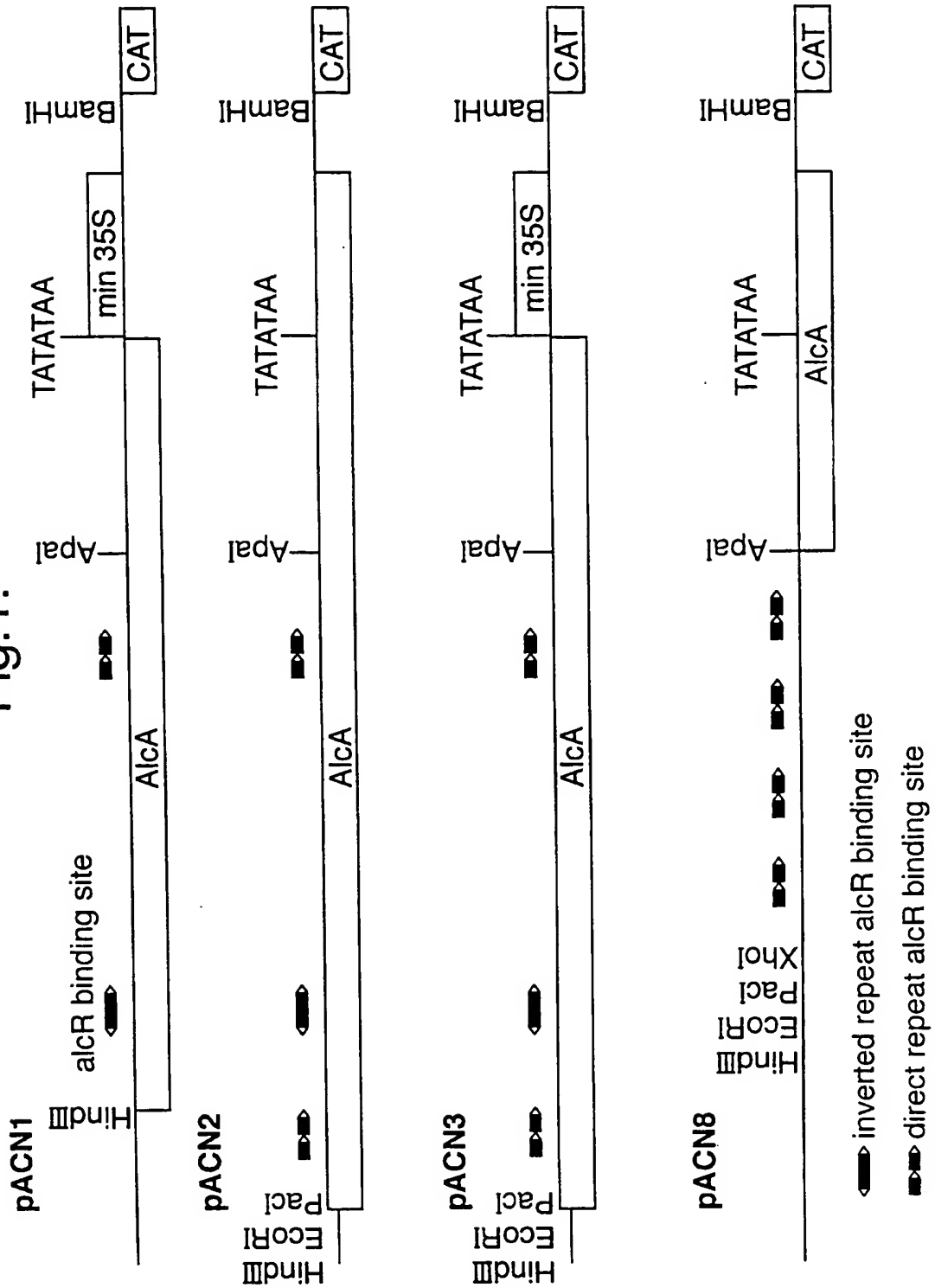

pACN1
HindIII
alcR binding site
ApaI
TATATAA
min 35S
BamHI
CAT
AlcA
pACN2
HindIII
EcoRI
PacI
ApaI
TATATAA
BamHI
CAT
AlcA
pACN3
HindIII
EcoRI
PacI
ApaI
TATATAA
min 35S
BamHI
CAT
AlcA
pACN8
HindIII
EcoRI
PacI
XhoI
ApaI
TATATAA
BamHI
CAT
AlcA
inverted repeat alcR binding site
direct repeat alcR binding site

Fig.2.

alcR binding site = ◀▬▬ or ▬▬▶

AlcA in pACN1

gggatagttccgacctaggattggatgcatgcggaaccgcacgagggcggggcggaaattgacacac

cactcctctccacgcaccgttcaagaggtacgcgtatagagccgtatagagcagagacggagcactttctggtactgtccgcacgggatgtccgcacggagagccacaaacgagcg

gggccccgtacgtgctctcctaccccaggatcgcatccccgcatagctgaacatctatataaggaagttcatttcatttggagaggacgac (SEQ. ID. No. 1)

apa I

AlcA in pACN2

tcgatagttgtgatagttcccacttgtccgtccgcatcggcatccgcagctcgggatagttccgacctaggattggatgcatgcggaaccgcacgagggcggggcggaaattgacacac

cactcctctccacgcaccgttcaagaggtacgcgtatagagccgtatagagcagagacggagcactttctggtactgtccgcacgggatgtccgcacggagagccacaaacgagcg

gggccccgtacgtgctctcctaccccaggatcgcatccccgcatagctgaacatctatataaagacccccaaggttctcagtctcaccaacatcatcaacc (SEQ. ID. No. 2)

apa I

AlcA in pACN3

tcgatagttgtgatagttcccacttgtccgtccgcatcggcatccgcagctcgggatagttccgacctaggattggatgcatgcggaaccgcacgagggcggggcggaaattgacacac

cactcctctccacgcaccgttcaagaggtacgcgtatagagccgtatagagcagagacggagcactttctggtactgtccgcacgggatgtccgcacggagagccacaaacgagcg

gggccccgtacgtgctctcctaccccaggatcgcatccccgcatagctgaacatctatataaggaagttcatttcatttggagaggacgac (SEQ. ID. No. 3)

apa I

AlcA in pACN8

gaattcttaattaactcgagtccgcacgggatgtccgca

cggggtaccgtcgagtccgcacgggatgtccgcacggggtaccgcgagtccgcacgggatgtccgcacggggtaccgtcgagtccgcacgggatgtccgcacggggtaccgtcgag

gggccccgtacgtgctctcctaccccaggatcgcatccccgcatagctgaacatctatataaagacccccaaggttctcagtctcaccaacatcatcaacc (SEQ. ID. No. 4)

apa I

Fig.3.

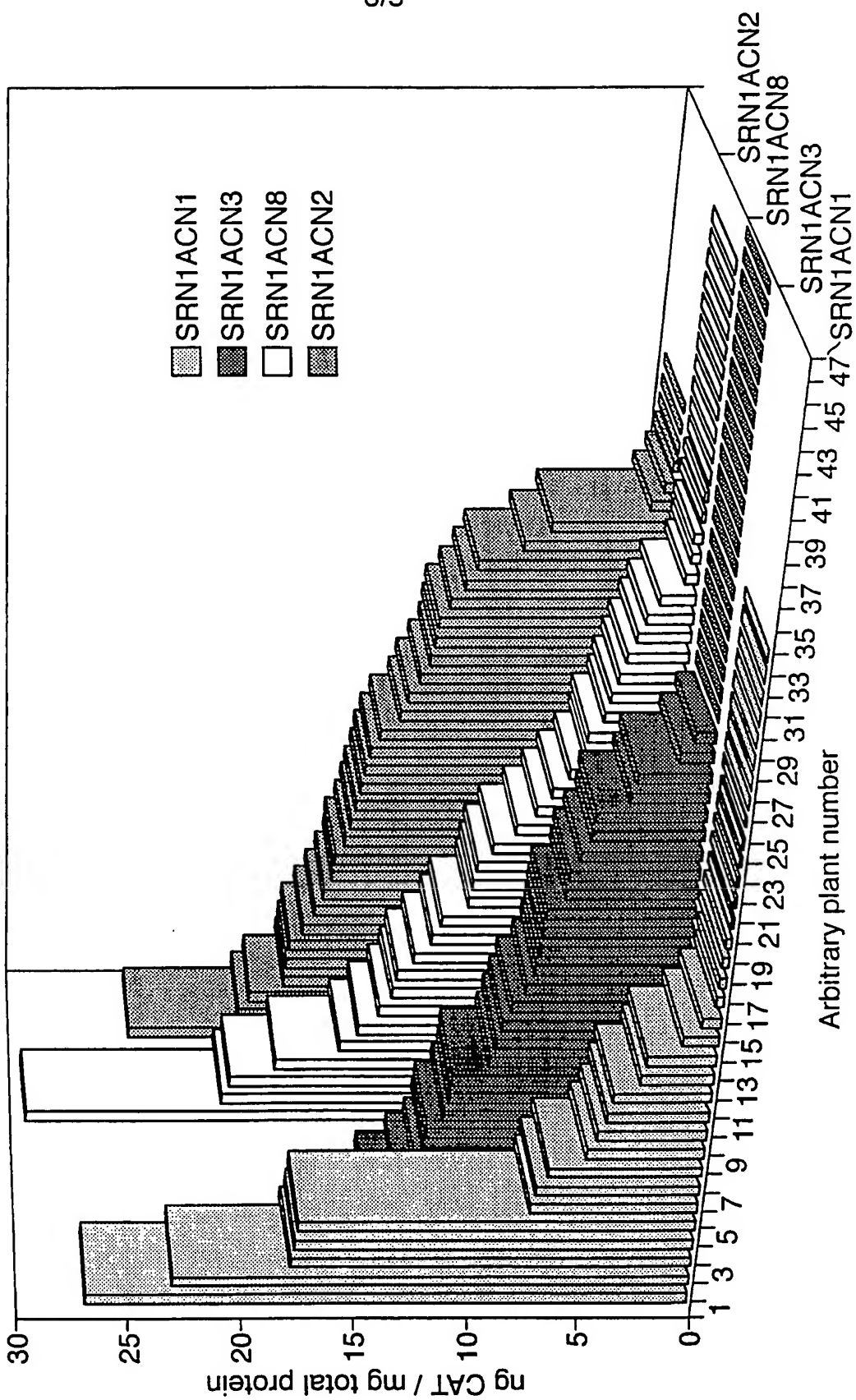

SRN1ACN1
SRN1ACN3
SRN1ACN8
SRN1ACN2
ng CAT / mg total protein
0
5
10
15
20
25
30
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47
Arbitrary plant number
SRN1ACN2
SRN1ACN8
SRN1ACN3
SRN1ACN1

Fig.4.

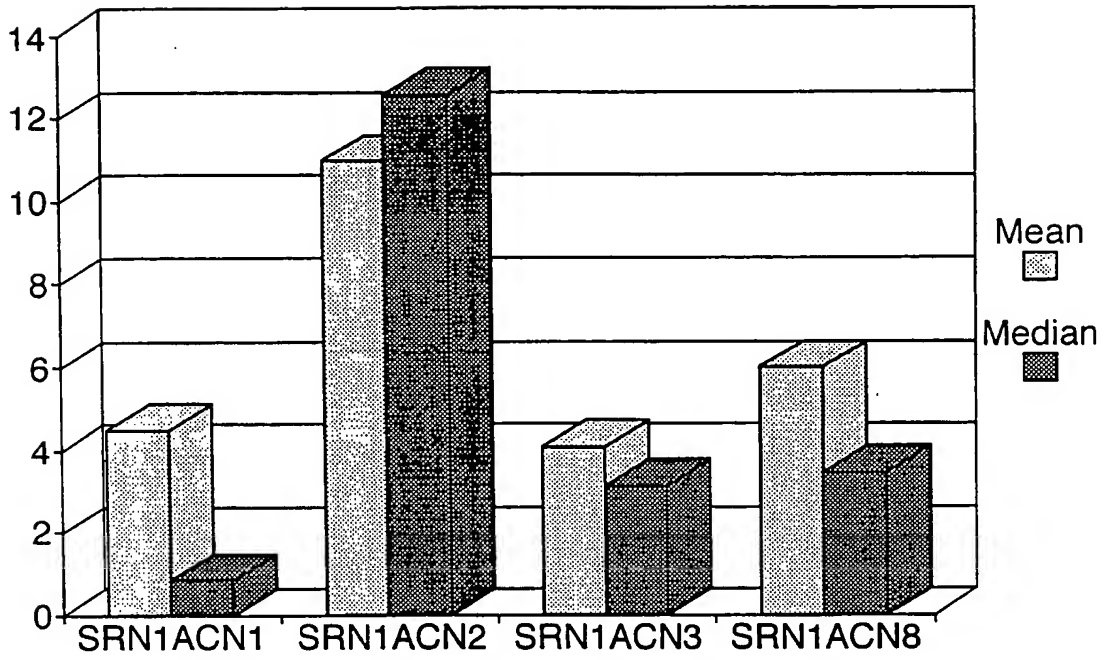

14
12
10
8
6
4
2
0
SRN1ACN1
SRN1ACN2
SRN1ACN3
SRN1ACN8
Mean
Median

Fig.5.

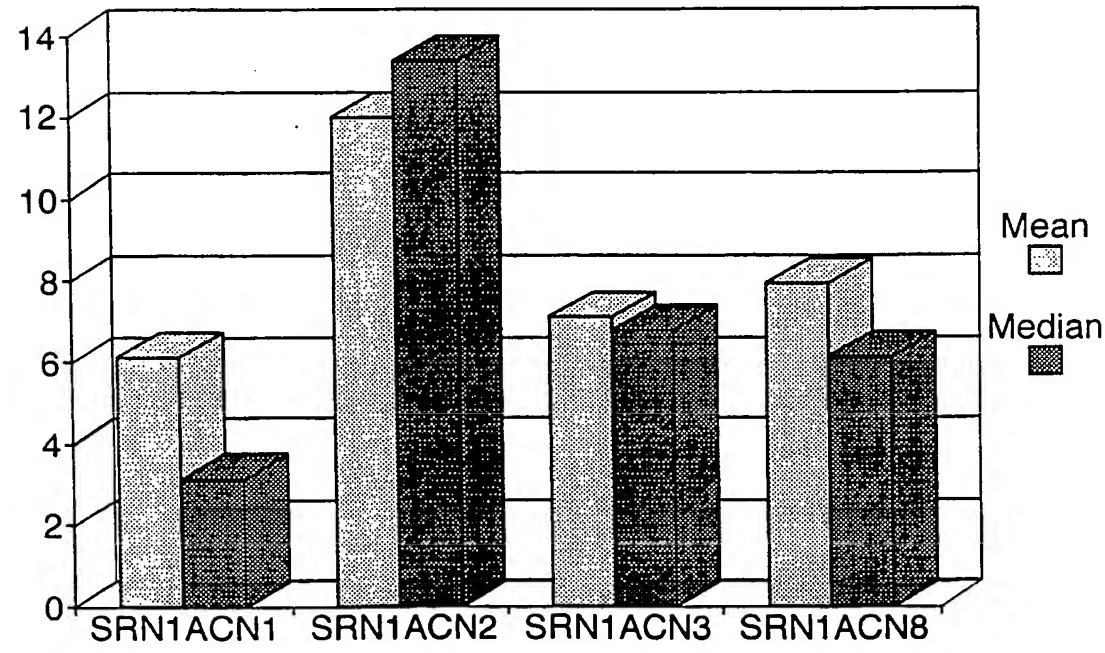

14
12
10
8
6
4
2
0
SRN1ACN1
SRN1ACN2
SRN1ACN3
SRN1ACN8
Mean
Median

Fig.6.

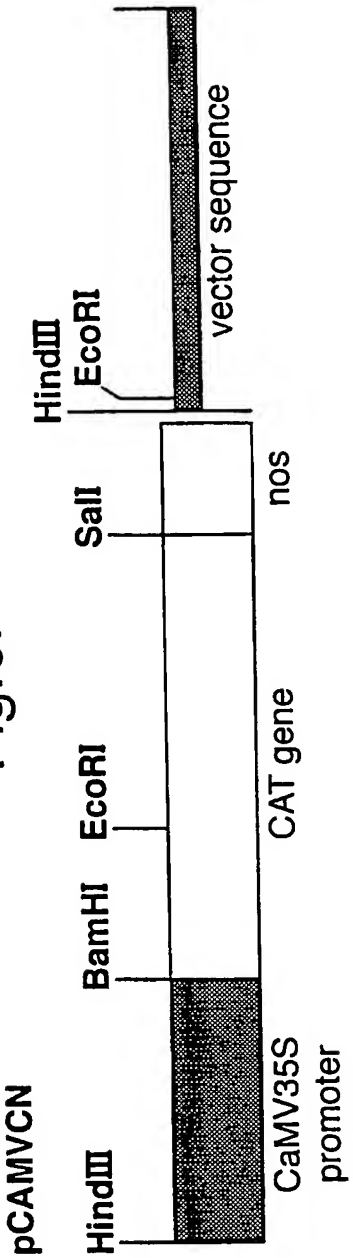


Fig.7.

From Lockington et al, Gene 33 (1985) 137-149